Impact of Chromosome 14q Loss on Survival in Primary Head and Neck Squamous Cell Carcinoma

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Abstract

We screened 73 primary head and neck squamous cell carcinoma (HNSCC) specimens for loss of heterozygosity (LOH) on chromosome 14q. Analysis of 20 polymorphic microsatellite markers identified 29 (40%) HNSCCs exhibiting LOH of 14q in at least one locus. Six tumors had probable monosomy of 14q, displaying allelic loss for all informative markers tested, and 23 demonstrated partial losses on 14q. Fine mapping with 1–10 additional markers revealed two poorly defined regions of loss (4–7 cM) at 14q13–21 and 14q31–32.1 in seven tumors. In 53 patients with previously untreated tumors treated with curative intent, LOH of 14q in these tumors correlated with poor survival. Compared to patients with tumors that retain heterozygosity of 14q, those with 14q LOH had a 3-fold increased risk of death in multivariate analysis (hazards ratio, 3.2; 95% confidence interval = 1.2–8.4). These data have confirmed a high frequency of chromosome 14q loss in HNSCC and suggest that LOH of any region on chromosome 14q is an indicator of poor outcome.

Introduction

New cases of HNSCC continue to afflict more than 50,000 Americans each year, resulting in about 12,000 deaths (1). Unfortunately, refinements in surgical technique and adjuvant therapy have not affected the 5-year survival rates for patients afflicted with HNSCC. Only recently have scientific efforts concentrated on unraveling the underlying genetic changes involved in the genesis and progression of HNSCC.

Preliminary evidence points to significant LOH on chromosome 14q in several tumor types, including endometrial carcinoma, epithelial ovarian carcinoma, renal cell carcinoma, HNSCC, and neuroblastoma (2–6). Recent studies have also reported an association between 14q loss and metastatic potential in colorectal carcinoma (7). In our initial allelotype of HNSCC, we observed allelic loss of 14q in 39% of primary tumors tested at one microsatellite locus (5). The genetic events in HNSCC, however, appear to be complex, as shown by LOH at 9p, 3p, 11q, 13q, and 17p in over 50% of cases and 4q, 6p, 8, and 19q in greater than 35% of cases tested (5). This is consistent with the observation that cellular transformation from a benign to malignant state arises through a series of genetic changes (8).

To further clarify the role of chromosome 14q in HNSCC progression, we examined 20 microsatellite loci in 73 paired normal and primary tumor samples for allelic loss. We have confirmed the high rate of allelic loss of 14q in HNSCC. Moreover, we found a strong correlation between those tumors exhibiting 14q loss and poor survival in affected patients.

Materials and Methods

DNA Extraction. Primary HNSCC tumors were obtained fresh at the time of surgical resection with prior consent from patients at The Johns Hopkins Hospital. Samples were fresh-frozen in liquid nitrogen or −80°C and carefully micro-dissected on a cryostat to separate out nonneoplastic cells. Those samples with greater than 70% tumor tissue were digested in SDS/protease K at 60°C for 6 h, followed by phenol-chloroform extraction and ethanol precipitation as described previously (9, 10). Blood was obtained by venipuncture from these patients, and lymphocyte DNA was isolated to be used as control DNA (5).

PCR Amplification. Normal and tumor DNA was analyzed for LOH by PCR amplification of polymorphic dinucleotide repeat sequences. All oligonucleotide primers listed in Fig. 1 were selected from the Genethon human genetic linkage map (11) and obtained from Research Genetics (Huntsville, AL). Prior to amplification, one primer was end-labeled with [γ-32P]ATP (DuPont NEN, Boston, MA) using T4 polynucleotide kinase (New England Biolabs, Boston, MA; Ref. 5).

Twenty-five ng of genomic DNA were used as PCR template in a 12.5-μl reaction including labeled primers and subjected to the following conditions: 30–35 cycles at 95°C for 30 s, 52–58°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 5 min. Amplification products were separated on denaturing 8% urea-polyacrylamide-formamide gels and visualized by autoradiography (12). LOH was scored (for informative cases) if the intensity of the signal of a single allele was markedly reduced (≥50%) in the tumor DNA by direct visual-
Fig. 1  Deletion map of chromosome 14q. The dinucleotide microsatellite markers and their relative positions on 14q are indicated on the left, along with the approximate distances between several markers. Black box, LOH; hatched box, retention of heterozygosity; white box, either normal or tumor DNA failed to amplify on initial amplification, or the marker was not critical for delineation of a deletion border; NT, noninformative. Minimal regions of loss are designated by bars on the right. Only the tumors demonstrating the smallest regions of loss are illustrated.

Survival Data. Survival after therapy was assessed for patients treated with curative intent (n = 53). Clinical outcome was derived from the hospital records. Individuals treated for palliation (6), those with recurrent disease at the time of study entry (13), and those who did not complete the planned course of therapy (1) were excluded to examine subjects with a similar outcome potential. Clinical parameters for these subjects are provided in Table 1. This cohort was assembled after treatment was completed; therefore, treatment was not uniform. Most individuals received combined surgery and postoperative radiation therapy. The completion of therapy marked the beginning of all time-to-event measurements. Event-time distributions for survival was estimated with the method of Kaplan and Meier (14) and compared using the log-rank statistic (13) or the proportional hazards regression model (15). The simultaneous effect of two or more factors was studied using the multivariate...
proportional hazards model. Covariates that were marginally significant ($P < 0.19$) in univariate analysis were entered into the multivariate regression model, and nonsignificant effects were removed in a stepwise fashion. Factors tested for prognostic value included the stage of disease, the presence or absence of cervical lymph node metastases, and patient age, gender, and heterozygosity status of chromosome 14q. Age was considered as a continuous variable. The effect of stage was analyzed by grouping patients with stages I and II disease and comparing them with patients with stages III and IV disease. All $P$s reported are two-sided. Computations were performed using SAS (16) or EGRET (17) statistical programs.

### Results and Discussion

Paired normal and tumor DNAs from 73 patients with invasive HNSCC were screened for LOH with 20 polymorphic microsatellite markers on chromosome 14q. Twenty-nine tumors (40%) exhibited allelic loss in at least one locus. The subset of tumors demonstrating partial deletions were then tested with 1–10 additional markers (Fig. 1) to further define the minimal region of loss. One group of tumors (T34, T277, T282, T285, and T231) showed deletions in the centromeric portion of 14q (Fig. 1). T34 displayed retention of heterozygosity at all informative loci on 14q except at $D14S47$, where LOH was observed. Although T34 potentially defines a very small deletion, we hesitate to define any border with a single tumor. T277 exhibits LOH at both $D14S47$ and $D14S255$ (Fig. 2). T282 demonstrates a deletion beginning outside the minimal area and extending to $D14S255$ (Fig. 1). Both T285 and T231 harbor deletions that span $D14S288$ and $D14S269$ (Fig. 1). This broader region spans a distance of approximately 4 cM (Ref. 18; Fig. 1).

Fine deletion mapping of 14q also reveals a region of distal loss on chromosome 14q31–32 (Fig. 1). T277, T282, T285, T231, T226, and T50 demonstrate deletions that include this region. T226 demonstrates a telomeric border at $D14S78$ and a centromeric border at $D14S45$. These data implicate the presence of a second locus of approximately 7 cM spanning chromosome 14q31–32 (18). T277, T282, T285, and T231 appear to harbor loss of both proximal and distal regions separated by an area of retention on chromosome 14q, suggesting the presence of two regions of loss. In addition, six tumors including T50 and T285 display complex deletion patterns with interrupted regions of loss and retention that do not correspond to the minimal regions delineated in the majority of samples. In addition, we do not like to define borders for a given region unless at least two tumors without rearrangements confirm the same breakpoint. Thus, these minimal regions (4–7 cM) remain poorly defined.

Although we cannot rule out other distinct areas of loss, complex genetic rearrangements are probably present in these outlying tumors. Certainly, other malignancies, such as T-cell leukemias and B-cell lymphomas, have been shown to harbor translocations and inversions on chromosome 14q (19, 20). In addition, the relative positions of microsatellite markers are susceptible to change, sometimes significantly, because linkage maps are continually revised. Tumors such as T285, T277, and T50, which show LOH in one marker outside the minimal areas of loss, may be clarified as these maps become increasingly accurate.

The clinical outcome of patients with primary tumors that exhibited loss of 14q in at least one locus was then compared to that of individuals whose tumors retained all markers. Survival was assessed for previously untreated patients who received a full course of treatment with curative intent. Fifty three (53)
Fig. 3 Overall survival for patients with head and neck squamous cell carcinoma categorized according to 14q status. Survival curves were generated using the Kaplan-Meier method. Representative 95% confidence intervals at several time points are provided (arrows), and the numbers of patients at risk at specific time points are shown below both sets of curves.

Table 2

<table>
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<th>Parameter</th>
<th>HR*</th>
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* HR, hazard ratio; CI, confidence interval.

patients were eligible for outcome analysis. Patient characteristics are shown in Table 1. Two individuals were lost to follow-up. Fifteen have died of disease; eight of these patients exhibited 14q LOH. Three are alive with disease, and five have died of unrelated causes. Follow-up for surviving patients ranged from 17-52 months, with a mean of 31 months. The proportion of patients surviving at various times is shown in Fig. 3. Table 2 shows the hazard ratios for death of patients grouped according to various clinical parameters in both univariate and multivariate models. Patients with tumors that had LOH of 14q demonstrate a 3-fold increased risk of death compared to those whose tumors retained heterozygosity for 14q. This difference remains statistically significant when controlled for stage and the related parameter of nodal status. In a recent study of 30 patients, 14q loss in renal cell carcinoma also correlated with poor clinical outcome as well as higher stage and histological grade (21).

It is possible that the retrospective nature of this study introduced bias in terms of treatment. As seen in Table 1, however, a disproportionate number of subjects with retention received primary radiation followed by surgical salvage. These individuals who experienced recurrence or persistence of disease would be expected to have a poorer overall survival, resulting in 14q retention being associated with a poor outcome. This was not seen. Certainly, our results should be corroborated in a larger prospective population treated uniformly.

Our initial allelotype of HNSCC revealed LOH of chromosome 14q in many primary tumors tested at one microsatellite locus. We have confirmed that 14q loss is a frequent genetic event in HNSCC, and our extensive mapping with at least 20 dinucleotide markers suggests at least two distinct areas of loss on 14q. Unfortunately, most tumors with partial deletions display a complex pattern of loss and retention, suggesting a series of noncontinuous interstitial deletions or complex chromosomal rearrangements. In support of two distinct regions of loss, we recently described two similar and potentially overlapping regions of loss on chromosome 14q in a large number of primary bladder cancers (22). These observations are consistent with the finding of more than one suppressor locus in most cases when monosomy is present. Perhaps the best example is monosomy of chromosome 9, where two distinct regions of loss are commonly implicated in the progression of bladder tumors and many other neoplasms (23).

When loss of 14q was correlated with clinical outcome, we were able to demonstrate a statistically significant difference in
survival between patients who exhibited LOH versus those who retained all 14q markers tested. Perhaps 14q alterations are a late progression event, or maybe these complex losses reflect the progressive accumulation of genomic instability. These advanced HNSCC neoplasms have certainly sustained multiple genetic alterations during their progression, and their accumulated losses have allowed us to fine map many tumor suppressor gene loci on multiple chromosomal arms. Further detailed mapping combined with cytogenetic analysis of these two distinct regions on chromosome 14q in more samples may further define the specific regions of loss and help locate putative suppressor gene loci. Analysis of 14q in premalignant lesions may elucidate the temporal relationship of these two distinct loss events and more accurately define the position that LOH of 14q occupies in the molecular progression of HNSCC and other neoplasms. Loss of a specific 14q locus may then be tested in large cohorts to better determine the overall impact of the molecular event in survival of HNSCC patients.

References
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